Mitochondrial DNA's from many vertebrates (mammals,1–7 birds,8 and amphibians9) and at least one invertebrate (sea urchin9) heretofore examined exhibit a remarkable structural similarity. All of these DNA's were found in the form of closed circular duplex molecules of approximately 5 μ in length, with an estimated molecular weight of 10⁹ daltons. However, the estimates of the DNA content per mitochondrion vary in different types of mitochondria from one or two9 up to 14 molecules8,7 of 10⁷ mol wt DNA.

In fungi, Luck and Reich10 isolated a linear DNA filament of 13 million daltons from Neurospora mitochondria, and the presence in mitochondria of at least two distinct density species of DNA has been indicated.11 In yeast mitochondria the presence of rather heterogeneous-size linear filaments at least 5 μ in length was observed by Sinclair et al.,12 but circular DNA species of varying length also present in the mitochondrial fraction were found to possess buoyant density identical to the corresponding nuclear DNA. This implies that the circular DNA might represent nuclear DNA contaminating the mitochondrial fraction. However, in separate experiments, Avers13 also observed in yeast mitochondria the presence of circular DNA molecules of up to 10.1 μ in length and estimated that an equivalent of 10 μ DNA molecule should be present per mitochondrion.

In previous studies14–15 with protozoa (Tetrahymena and Paramecium) and several plants, we found DNA contents of 300 to 500 million daltons per mitochondrion. Purified Tetrahymena mitochondrial DNA had a relatively uniform sedimentation coefficient of 41S16 and was believed to represent an intact form existing in vivo. However, we were unsure whether the 41S DNA assumed a circular or linear structure. Studies were therefore made to examine Tetrahymena mitochondrial DNA by the Kleinschmidt's protein monolayer spreading technique.17 For comparison, DNA's of plants and monkey liver mitochondria were also included in these studies.

Materials and Methods.—Tetrahymena mitochondria and mitochondrial DNA used in the present studies were isolated from ST strain of Tetrahymena pyriformis by the method described previously.16 The isolated mitochondrial DNA showed a density of 1.686 gm cm⁻³ and less than 3% nuclear DNA contamination.

Monkey (Macaca mulata) mitochondria were isolated from the liver (a courtesy of Dr. L. Mastroianni, Pennsylvania University Hospital) which was homogenized under aseptic conditions with a mortar and pestle in 0.25 M sucrose. The homogenate was centrifuged at 180 g for 6 min, and the resulting supernatant fluid was centrifuged at 5000 g for 6 min. The mitochondrial pellet was resuspended in the same sucrose medium and washed three times by repeated centrifugation at 10,000 g for 15 min. The final mitochondrial pellet was treated with DNase (16 μg/ml at 0.05 M MgSO₄), and DNA was isolated as described.14,16

Purified DNA was prepared for electron-microscopic examinations by the method of Kleinschmidt and Zahn.17 To examine purified DNA, a DNA-cytochrome c (0.01%) mixture in 1 M ammonium acetate was layered over a water-hypophase surface. To examine DNA released by osmotic disruption, isolated mitochondria suspended in cold
4 M ammonium acetate containing 0.01% cytochrome c were layered over chilled water-hypophase surface, as described by Nass. DNA-protein film was picked up on a carbon-formvar coated copper grid (200-mesh). The specimens were dried in air and shadowed at a grazing angle of 7° in a vacuum evaporator at 0.03 μ Hg or less, while they were continuously rotated on a small turntable. Shadowing with platinum-iridium (80:20, Fullam) was repeated until DNA filaments became clear for electron-microscopic examinations. Electron micrographs were made with a Philips 200 electron microscope at magnification of 5400. A diffraction grating replica (Fullam, 54,864 lines/inch) was used to calibrate the magnifications. Contour length was measured with a map measurer on positive prints at over-all magnifications of at least 34,000.

Results and Discussion.—Purified DNA which was previously estimated to be 41S showed a relatively uniform length; and for 103 linear filaments ranging from 16.1 to 19.3 μ, the mean length was 17.6 μ ± 0.077 (SE). The frequency distribution of the lengths of these filaments is shown in Figure 1. A relatively large filament length makes it difficult to score many measurable molecules. Some were found in rosette or flowerlike forms, most of which showed two free ends. Although their lengths were difficult to measure, measurements made on several of these molecules were comparable to that obtained from a linear extended filament. One linear filament was 22 μ long, and two others had the minimum length of 22 μ, but the complete filament length was not measurable because their ends were lost in the metal grid. Also scored were 15 additional filaments which were smaller than 16 μ ranging from 9 to 15.3 μ. The origin of these molecules was not clear, but they may represent broken pieces since there is no definite maximum in length distribution. Also very small-molecular-weight DNA's were seen in electron micrographs and also in sedimentation analysis. No circles have been identified.
DNA released from osmotically disrupted mitochondria was also examined. Since membranous fragments, presumably of mitochondrial cristae, are abundant and since portions of DNA filaments were often buried under these fragments (Fig. 2), it was difficult to obtain photographs of a number of measurable filaments. However, in other photographs 16 filaments were measurable, and the distribution of their lengths is presented in Table 1; the calculated mean length was $17.6 \mu \pm 0.15$. Two additional filaments (their lengths 10 and 13 \(\mu\)) were also found, but they were considered as broken pieces.

As mentioned above, there are many DNA filaments which were connected with masses of mitochondrial membranes or fragments. However, there is no single site of association for DNA on the membranes, and DNA filaments radiate from all sides of broken mitochondrial debris (Fig. 3). Sometimes, clusters of DNA filaments free from the remnants of mitochondrial membranes were observed. Since the mitochondrial density on the grid was low, it is unlikely that

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**Fig. 2.—**Electron micrograph of DNA released from osmotically disrupted mitochondria. DNA was often found in association with clumped cristae or membranous fragments. Magnification \(\times21,330\).
TABLE 1. Length distribution of DNA released from disrupted Tetrahymena mitochondria.

<table>
<thead>
<tr>
<th>Length (μ)</th>
<th>Number of filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.5</td>
<td>1</td>
</tr>
<tr>
<td>16.7</td>
<td>1</td>
</tr>
<tr>
<td>17.2</td>
<td>4</td>
</tr>
<tr>
<td>17.5</td>
<td>1</td>
</tr>
<tr>
<td>17.6</td>
<td>3</td>
</tr>
<tr>
<td>18.0</td>
<td>2</td>
</tr>
<tr>
<td>18.1</td>
<td>1</td>
</tr>
<tr>
<td>18.4</td>
<td>1</td>
</tr>
<tr>
<td>18.7</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>16</strong></td>
</tr>
</tbody>
</table>

The mean length = 17.6 μ, and the standard error = 0.15.

Fig. 3.—Electron micrograph of DNA (D) released from osmotically disrupted mitochondria. Mitochondrial outer membrane (O), the shape of which is well preserved, and clumped cristae (C) are seen. DNA filaments radiate from many points of disrupted mitochondria. The points of association of DNA to cristae are indicated by arrows. Magnification ×16,827.
these masses of DNA resulted from aggregations of DNA filaments liberated from different mitochondria. The amount of DNA seen in each of those clusters of filaments is large, but many ends are clearly visible. For example, 17 ends can be identified in Figure 4, but 14 are connected to the DNA mass in the center. Since the total length of the cluster of filaments visible in this picture corresponds to roughly 110 \( \mu \), each of seven linear filaments in the cluster is a linear filament of at least 15 \( \mu \) in length. In another picture, at least 16 ends with a clustered DNA corresponding to 147 \( \mu \) were identified.

The above results suggest that *Tetrahymena* mitochondrion contains over seven molecules of an average 17.6-\( \mu \)-length DNA. The molecular weight of each molecule, calculated on the assumption of B configuration with a mass per unit length of 1.92 million daltons/\( \mu \),\(^{18}\) is 33.8 million daltons. It was previously estimated that the *Tetrahymena* mitochondrion contained approximately \( 3.7 \times 10^{-10} \) \( \mu \)g DNA which is equivalent to a DNA piece of \( 2.4 \times 10^8 \)}
The present results are in good agreement with this estimate. However, some discrepancy is apparent between the molecular weight estimates from the sedimentation value of 41S (approximately 42 to 45 million daltons) and by the present method. This may be due to an experimental error in the determination of the sedimentation value resulting from the intrinsic difficulties involving the determination of high-molecular-weight DNA.

In all these studies, we have never observed circular DNA. In order to increase the resolution of detecting circular species, CsCl density gradient centrifugation in ethidium bromide was also performed with mitochondrial lysate and purified DNA as well as whole-cell lysate. In no case did we find a secondary band attributable to circular DNA. We conclude, therefore, that *Tetrahymena* mitochondria isolated from an exponential cell culture contain only linear DNA molecules.

The melting temperature (Tm) of *Tetrahymena* mitochondrial DNA in 0.195 N NaCl, defined as that temperature which gives 50 per cent hyperchromicity of 260 nm absorbancy, was previously shown to be 79.5°C. At 75°C, only 3 to 4 per cent of the hyperchromicity was attained. If mitochondrial DNA consisted of small molecules joined by H-bonding of noncovalently linked complementary single strands of approximately 3 to 4 per cent of the total length, DNA heated at 75°C and fast-cooled might break into smaller subunits. The results of such an experiment showed that the DNA strands recognizable by electron microscopy are still large; the mean length for eight molecules was 17.2 µ ± 0.11. However, one circular molecule of 0.3-µ length, the only circular DNA observed in many preparations of *Tetrahymena* mitochondrial DNA, was found.

We extended electron-microscopic studies on mitochondrial DNA's from other sources. Preliminary data show that DNA isolated from mung bean hypocotyl mitochondria is of varying lengths from 12 to 18 µ, and *Paramecium* mitochondrial DNA varies from 8 to 16 µ, but length distributions of both of these linear filaments are not yet available.

On the other hand, about 50 per cent of the total mass of monkey liver mitochondrial DNA was in the form of a ring structure of a mean length, 5.5 µ ± 0.05; the distribution of length is shown in Figure 5. In addition, one ring-shaped molecule each of 2.3, 7.6, 11, and 15 µ in length was observed (Fig. 6). Although groups of circles representing multiples of the basic length were previously observed in HeLa cells and human leukocyte preparations, not all of...
these sizes can be explained on the same basis. The possibility of contamination with other types of DNA cannot be excluded.

The studies presented here demonstrate the great variation of size in mitochondrial DNA's of various organisms. It is important to emphasize here that the *Tetrahymena* mitochondria contain DNA of approximately 34 million daltons, which is 3 to 4 times that of DNA size found in other organisms studied to date.

There is a possibility that all other mitochondria may require at least a comparable mass length of DNA to carry out the essential function within mitochondria, since few biochemical and physiological differences have been detected in mitochondria of various organisms. Therefore, it is not unreasonable to postulate that there are more than three linkage groups present in those mitochondria which contain a relatively small-molecular-weight DNA.

Because of the existence of multiple-length circular DNA molecules in mitochondria of HeLa cells as well as human leukocytes, there is an alternative
possibility for explaining the present situation, i.e., *Tetrahyymena* mitochondrial DNA might represent a multiplex form. However, this is considered unlikely, since the hybridization value of mitochondrial DNA with ribosomal RNA and also in vitro RNA synthesis data are more compatible with the DNA molecule of about 30 million daltons or higher.21, 22

**Summary.**—Unlike mitochondrial DNA of other organisms, DNA purified from *Tetrahyymena* mitochondria has a linear structure of a mean length of 17.6 \( \mu \pm 0.077 \), as revealed by electron microscopy. The mean length of DNA released from osmotically disrupted mitochondria was 17.6 \( \mu \pm 0.15 \). A cluster of seven or more strands of DNA of this size was released from a single mitochondrion.

Electron-microscopic studies of purified DNA from *Paramyema* and of mung bean hypocotyl mitochondria showed only the presence of a linear filament with a size comparable to that of *Tetrahyymena* mitochondrial DNA, whereas a large portion of monkey (*Macaca mulata*) liver mitochondrial DNA was circular with a mean length of 5.5 \( \mu \pm 0.04 \).

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4 Radloff, R., W. Baner, and J. Vinograd, these PROCEEDINGS, 57, 1514 (1967).
5 Nass, M. M. K., these PROCEEDINGS, 56, 1215 (1966).
10 Luck, D. J. L., and E. Reich, these PROCEEDINGS, 52, 931 (1964).
11 Reich, E., and D. J. L. Luck, these PROCEEDINGS, 55, 1600 (1966).
13 Avers, C. J., these PROCEEDINGS, 58, 620 (1967).